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DIVERGENT RESERPINE EFFECTS ON AMFONELIC ACID AND AMPHETAMINE STIMULATION OF SYNAPTOSOMAL DOPAMINE FORMATION FROM PHENYLALANINE*

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Abstract—Some effects of a non-amphetamine central stimulant, amfonelic acid, on the synaptosomal (P_2) formation and release of dopamine being produced continuously from [^{14}C]phenylalanine substrate have been determined. How reserpine action may modify the effects of amfonelic acid and those of amphetamine (the latter was included for comparative purposes) was also examined. For these studies, P_2 preparation from rat caudate nuclei was incubated with [^{14}C]phenylalanine with and without various drug additions, and the particulates were separated from the medium after incubation. The separated fractions were analyzed for labeled dopamine and for the synaptosomal content of the labeled substrate. Of the total labeled dopamine formed, the fraction that was present in the medium was determined and taken as a measure of the spontaneous release (no drug addition) or its enhancement by any addition. Amfonelic acid and amphetamine (0.91–18.2 μM) comparably stimulated the synthesis and release of [^{14}C]dopamine. The addition of reserpine (1.8 μM) alone had an inhibitory effect on total synthesis and a stimulatory one on release. In the presence of reserpine, the synthesis stimulation by amphetamine was maintained or accentuated, but amfonelic acid induced an inhibitory effect additive to that due to reserpine alone. The release stimulations by amphetamine and amfonelic acid were comparable in the absence as well as in the presence of reserpine. Following reserpine pretreatments at 24 and 2 hr, amphetamine (9.1 μM) markedly stimulated, but AA (9.1 μM) affected nonsignificantly, the dopamine synthesis. The pretreatments did not abolish the release-enhancing effect of either stimulant. None of the drug additions resulted in a significant alteration of the particulate [^{14}C]phenylalanine substrate level. In summary, the results show that amfonelic acid, like amphetamine, may release continuously forming synaptosomal dopamine and stimulate dopamine synthesis. However, the synthesis stimulation by amfonelic acid, but not that by amphetamine, may be abolished by reserpine action, either *in vitro* or *in vivo*. The results suggest that, although amphetamine may stimulate by releasing the newly forming dopamine pool, a significant amfonelic acid action may be on the catecholamine storage system, and synaptosomal dopamine synthesis may be under the controlling influence of both the newly forming amine and the vesicular stores.

Amfonelic acid (AA) is a potent non-amphetamine stimulant which mimics the central effects of amphetamine (AMT). The latter exerts its behavioral effects via catecholamines, particularly dopamine (DA), by releasing the newly synthesized amine and blocking its reuptake [1–3]. AA-induced behavioral effects [4, 5] may also be mediated via catecholamines and Shore [6] after studying the effect of AA on brain DA and its metabolites, has suggested that this drug may affect a pool of DA. The mechanism of action of AA, however, is likely to differ from that of AMT in some way since it is well known [1] that the central stimulant actions of AMT are not attenuated by reserpine (Res) pretreatment which, however, blocks AA stimulation [4].

The nature of the action of AA on dopaminergic neurones is not clearly understood, and little is known as to how Res, a potent biogenic amine depleting agent, may affect that action. The synaptosomal preparation (P_2) of brain is a useful *in vitro* model of a neuronal system [7, 8], but no report has been published thus far, to our knowledge, investigating how Res may influence the actions of AA on the synaptosomal formation of DA and its release. In the present study we have examined how AA, and AMT for comparative purposes, may affect synaptosomal synthesis of DA and its release and the influence of Res on such effects of these stimulants. DA formation from phenylalanine in brain has been shown by several studies [9–11] to respond markedly to a number of psychotropic drugs; we have used [^{14}C]phenylalanine in our present study as the substrate for synaptosomal DA formation. Our preliminary communication describing the method and some of the observed effects of AMT, AA and Res on such DA formation has recently been published [12].

MATERIALS AND METHODS

AA was a gift from the Sterling–Winthrop Research Institute, Rensselaer, NY; Res was Serpasil (Ciba Pharmaceuticals, Summit, NJ). AMT was used as D-amphetamine sulfate; expressed concentrations are of free AMT.

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Table 1. Reserpine effects *in vitro* on AMT and AA stimulation of synaptosomal DA synthesis from [^{14}C]phenylalanine*

Addition (μM)	N	[^{14}C]DA (total)		Release index		Particulate [^{14}C]Phe	
		−Res	+Res	−Res	+Res	−Res	+Res
None	26	100% (106.7 ± 8.9)†	100% (32.8 ± 3.3)	1.00 (21.0 ± 1.7)	1.00 (67.6 ± 2.3)	100% (1470.7 ± 29.6)	100% (1387.2 ± 33.9)
AMT (0.91)	4	182.7 ± 19.8‡	125.8 ± 11.7	1.18 ± 0.23	1.37 ± 0.03§	99.1 ± 6.8	104.0 ± 4.1
AMT (4.5)	4	187.2 ± 27.6‡	231.6 ± 24.4§	2.09 ± 0.38§	1.39 ± 0.04§	92.0 ± 4.9	105.3 ± 3.9
AMT (9.1)	8	235.7 ± 25.3§	314.3 ± 26.7§	2.00 ± 0.19§	1.39 ± 0.05§	105.5 ± 3.3	97.4 ± 5.0
AMT (18.2)	5	234.5 ± 23.6§	299.4 ± 39.8§	2.55 ± 0.31§	1.40 ± 0.04§	95.6 ± 4.6	101.2 ± 3.0
AA (0.91)	4	146.2 ± 7.9‖	46.6 ± 7.5‖	1.94 ± 0.28§	1.39 ± 0.05§	95.2 ± 5.3	102.0 ± 2.3
AA (4.5)	5	210.0 ± 37.7§		2.01 ± 0.37§		101.1 ± 3.1	
AA (9.1)	6	238.0 ± 20.0§	53.6 ± 3.8‖	1.85 ± 0.25§	1.29 ± 0.07‡	111.1 ± 4.2	100.5 ± 5.6
AA (18.2)	5	178.2 ± 9.8§	51.2 ± 5.1‖	1.76 ± 0.22§	1.29 ± 0.04§	91.5 ± 3.7	108.0 ± 4.7

* The standard incubation mixture contained P_2 from 9.5 mg caudate tissue suspended in Tris buffer (pH 7.4) containing salts, glucose, sucrose and pargyline in a final volume of 210–220 μl (see Materials and Methods). The additions were as 10 μl of aqueous solutions; Res when added (+Res) was 1.8 μM. The [^{14}C]phenylalanine substrate concentration and specific radioactivity were 6.1 μM and 209.0 nCi/nmole, respectively. After a 10-min incubation (37°) the mixtures were filtered and the separated fractions were analyzed. The release index was calculated by dividing the medium/total ratio of labeled DA observed in the presence of an addition by the same ratio in the corresponding control (no addition) sample. The results are expressed as the means of a number (N) of experiments ± the S.E.M. The significance of the difference from the corresponding control (no addition) is indicated by the P values given in the footnotes with the symbols ‡, §, ‖.

† The actual values within parentheses are: [^{14}C]DA and [^{14}C]Phe as pmoles per 100 mg per 10 min; the release as medium [^{14}C]DA/total [^{14}C]DA × 100.

‡ $P < 0.005$.

§ $P < 0.001$.

‖ $P < 0.05$.

The methods described below are the same as those detailed in our preliminary communication [12] with slight modifications. Female Wistar rats (150–200 g, Charles River Co., Wilmington, MA) were decapitated and the caudate nuclei (including putamens) were dissected. Following the homogenization of the tissue (10% in ice-cold 0.32 M sucrose solution containing 10 μM calcium chloride), the synaptosomal–mitochondrial fraction (P_2) was prepared by differential centrifugation at 0–4° as described by Whittaker and Barker [7]. The initial crude nuclear fraction was washed once by resuspending it in 0.32 M sucrose; the washing was combined with the supernatant fraction (S_1) before the final centrifugation to obtain P_2. The P_2 pellet was suspended in Tris buffer (10 mM, pH 7.4) containing NaCl (125 mM), KCl (5 mM), $MgCl_2$ (15 mM), pargyline (0.08 mM), glucose (10 mM), and sucrose (0.32 M) which has been observed by Sperk and Baldessarini [13] to stabilize brain synaptosomes. The suspension was incubated in open test tubes at 37° after the addition of [^{14}C]phenylalanine (L-phenylalanine[U-^{14}C], specific radioactivity 350–400 mCi/mole, New England Nuclear Corp., Boston, MA), and cold L-phenylalanine was added as necessary to make up the final substrate concentrations listed in Results. The addition of various agents listed in Results was as the aqueous solution of each compound in a 10 μl volume. The final volume of the incubation mixture was 210–220 μl containing synaptosomal preparation representing 9.5 mg of original whole caudate tissue weight. At the termination of the incubation period, the synaptosomal particles were separated from the incubation medium. For this purpose 2.0 ml of ice-cold mixture identical in composition with that of the incubation medium was added to the mixture to stop the reaction. The particulates were immediately separated from the medium by pouring the incubation mixture on an 0.8 μm size Millipore filter under suction. The entire process of diluting and separating the particulates from the medium was completed within 5–10 sec, thus assuring a negligible exchange of label between the fractions. The collected particulates were further washed by an additional 2.0 ml of the ice-cold medium, and the filter was then dropped in 2.0 ml of cold 0.4 N perchloric acid containing 0.1% Triton X-100. The acid-extracted (2x) samples of the particulate fraction and of the separated medium as well were then analyzed for labeled DA by absorption on activated alumina and subsequent 0.5 N acetic acid elution as described before [14]. The recovery of DA was 79 per cent; data were not corrected for recovery. The purified samples of DA were assayed for ^{14}C by liquid scintillation counting. The DA samples from the blanks, which were the same as the control (no addition) samples except that they were filtered without the incubation at 37°, counted 6.7 ± 1.3 per cent (N = 7) of the control (no addition) samples. The effluent and washings obtained from the alumina absorption procedure contained the labeled phenylalanine substrate present in the acid extracts. The level of ^{14}C in the effluent fraction from the synaptosomal acid extract analysis was obtained by counting an aliquot, and the radioactivity was taken as an index of the synaptosomal uptake of the labeled substrate. The substrate uptake and the product formation were expressed as picomoles of substrate or product per 100 mg (of whole caudate tissue equivalent of the P_2 sample) per 10 min (of incubation). The radioactivities of the fractions were first converted to pCi per 100 mg per 10 min and further divided by the final specific radioactivity (pCi/pmoles) of the labeled substrate to arrive at the pmoles per 100 mg per 10-min values. The final specific radioactivity was calculated from the radioactivity added and the final concentration which was the total of labeled, endogenous, and any added cold substrate. The substrate and other concentrations expressed in Results were based on the final incubation volume. Endogenous phenylalanine was assayed by a method consisting of ion-exchange analysis, gas chromatography, and mass spectrometry, the details of which will be published elsewhere. No correction for recovery was applied to the data reported. Calculations of the standard error of the mean (± S.E.M.) and the level of significance were according to Snedecor and Cochran [15], as described for Student's t-test.

RESULTS

The results in Table 1 show the total (the sum of particulate and medium fractions) synthesis of [^{14}C]DA and the particulate level of the labeled substrate in the presence of added AMT, AA, and Res. These data are expressed as the percentage of the corresponding control value of pmoles per 100 mg per 10 min given within parentheses. Table 1 also summarizes the stimulating effects of the drugs on the release of synaptosomally formed [^{14}C]DA into the incubation medium. Such stimulation is expressed by the release index, indicating the release above that in the corresponding control sample. The index was derived by dividing the medium/total ratio of [^{14}C]DA in the presence of an addition by that ratio from the control, given within the parentheses.

As the results indicate, the total DA formation from [^{14}C]phenylalanine was stimulated in the presence of AMT and AA. The extent of stimulation by AA, at any of the various concentrations (0.91–18.2 μM) tested, was generally comparable to that induced by AMT at the same concentration. The stimulation of DA synthesis by AA was highest (238.0 per cent of control; $P < 0.001$) at 9.1 μM; at 0.91 μM it was 146.2 per cent ($P < 0.05$). Similarly, AMT increased [^{14}C]DA synthesis to a maximum value of 235.7 per cent ($P < 0.001$) at 9.1 μM; the lowest concentration tested (0.91 μM) resulted in a value of 182.7 per cent ($P < 0.005$) of the control.

The results in Table 1 also show the effects of AMT and AA on the synaptosomal [^{14}C]DA synthesis with 1.8 μM Res in the incubation medium. As the data show, AMT elevation of [^{14}C]DA synthesis was observed in the presence of Res and the total synthesis ranged from 125.8 per cent of the control (Res alone) at the lowest concentration (0.91 μM AMT) to 314.3 per cent ($P < 0.001$) at 9.1 μM AMT. At each of the AMT (Res plus) concentrations except 0.91 μM, the degree of stimulation appeared to be higher than that observed with AMT alone at the corresponding concentration. The addition of Res alone significantly ($P < 0.001$)

Table 2. Actions of AMT and AA on synaptosomal DA formation from [^{14}C]phenylalanine following reserpine pretreatment *in vivo**

Addition (μM)	N	[^{14}C]DA (total)	Release index	Particulate [^{14}C]Phe
None	8	100% (64.2 ± 8.5)	1.00 (52.6 ± 3.5)	100% (1344.9 ± 57.7)
AMT (9.1)	7	439.6 ± 59.9 $P < 0.001$	1.87 ± 0.11 $P < 0.001$	98.8 ± 2.3 NS†
AA (9.1)	7	117.6 ± 17.5 NS	1.63 ± 0.11 $P < 0.001$	100.9 ± 3.6 NS
Res (1.8)	7	98.2 ± 13.7 NS	1.12 ± 0.08 NS	98.6 ± 3.2 NS

* Rats were pretreated with Res at 24 hr (5.0 mg/kg, i.p.) and at 2 hr (2.5 mg/kg) before the experiment. The experimental details are described under Table 1.

† NS, not significant.

inhibited [^{14}C]DA synthesis to 30.7 per cent, an inhibition that we reported before [12] and suggested was due to an enlarged cytoplasmic amine pool inhibiting phenylalanine hydroxylation. Unlike the AMT effect in the presence of Res, however, the co-addition of AA and Res resulted in [^{14}C]DA synthesis reduction greater than that due to Res alone. AA concentrations of 18.2, 9.1 and 0.91 μM in the medium reduced [^{14}C]DA synthesis to 51.2 per cent ($P < 0.05$), 53.6 per cent ($P < 0.05$) and 46.6 per cent ($P < 0.05$) of the control (Res plus) value, respectively.

The release indices summarized in Table 1 indicate that both AMT and AA increased the release of DA synthesized continuously from [^{14}C]phenylalanine beyond that of spontaneous release. Although the addition of these two drugs at the higher concentrations resulted in comparable indices ranging between 1.76 and 2.55 ($P < 0.001$), 0.91 μM AA appears to have a more potent releasing action. Upon the addition of Res to the medium, the total release of [^{14}C]DA in the presence of AMT and AA was even higher, with indices between 4.15 and 4.51 ($P < 0.001$) expressed in relation to the drug free spontaneous release. Both AMT and AA caused additional stimulation of release beyond that due to Res, and the indices were between 1.29 and 1.40 ($P < 0.005$ to $P < 0.001$) relative to the release observed with Res alone.

We have also determined the particulate level of [^{14}C]phenylalanine which may be taken as an indicator of the synaptosomal substrate uptake. As the data show (Table 1), the substrate uptake was not significantly affected by the various additions of AMT and AA with and without Res.

Brief exposure of synaptosomes to Res during sample incubations may not cause the marked decrement of endogenous catecholamine levels that occurs after Res treatment in behavioral studies on AA and Res interaction [4]. To deplete the endogenous catecholamines, we pretreated rats with 5.0 mg/kg Res (i.p.) at 24 hr and 2.5 mg/kg at 2 hr before the preparation of P_2. According to Kuczenski [16], such pretreatment leads to a large (> 90 per cent) depletion of caudate tissue DA. The P_2 fractions from such pretreated rats were used for the determination of AMT and AA effects on the synaptosomal DA synthesis from [^{14}C]phenylalanine. The data (Table 2) show that 64.2 pmoles per 100 mg per 10 min of [^{14}C]DA were formed in the control (no addition) sample, which is 60.2 per cent ($P < 0.0025$) of the [^{14}C]DA formed from the incubation of untreated P_2 (Table 1; no addition). The results in Table 2 also show that, in the Res pretreated P_2, AMT (9.1 μM) strongly stimulated (439.6 per cent of the control, $P < 0.001$) [^{14}C]DA formation, whereas AA was ineffective (117.6 per cent; NS). The release indices show, however, in contrast with the divergent effects of AMT and AA on the total [^{14}C]DA formation, that both of the agents were effective in stimulating the release of DA formed from [^{14}C]phenylalanine. The AMT release index was 1.87 ± 0.11 ($P < 0.001$ vs no addition) and the AA index was 1.63 ± 0.11 ($P < 0.001$ vs no addition). In some of our experiments, 1.8 μM Res was added to the pretreated P_2 samples. The results (Table 2) show little effect of that addition either on the total synthesis (98.2 per cent of the no addition sample; NS) or on the synaptosomal release (index 1.12 ± 0.08; NS). The particulate level of [^{14}C]phenylalanine shown in Table 2 indicates that the uptake of the labeled substrate was not significantly affected by the addition of AMT, AA, or Res. Also, the substrate uptake values in these experiments were very similar to those from the incubations of P_2 from untreated animals (Table 1).

DISCUSSION

The results from various laboratories as reviewed by Moore [1] indicate that AMT central stimulation is Res resistant and that AMT may induce the release of newly formed nerve ending DA, the likely mediator of the stimulant action. In harmony with these results, our present data (Tables 1 and 2) indicated that AMT may release newly synthesized synaptosomal DA from [^{14}C]phenylalanine. Our present results also indicate, as do those of others [17, 18] using labeled tyrosine as the DA precursor, that AMT may stimulate synaptosomal DA synthesis. Such stimulation was suggested [17] to be due to the AMT-induced release of an inhibitory synaptosomal catecholamine pool at the site of the amino acid hydroxylation. Our data further show,

consistent with the inability of Res to attenuate AMT behavioral stimulation, a Res resistance of the AMT action that increases the synaptosomal release of DA and also its formation; such Res resistance of AMT action on DA formation has been reported before [18].

Our results indicate comparable effects of AMT and AA in increasing the synthesis as well as the release of the newly forming [^{14}C]DA. It would therefore seem that the mechanism of action of AA on synaptosomal DA may be similar to that of AMT. The results of incubating in the presence of Res, however, suggest some divergence of AMT and AA actions. Although the degree of synthesis stimulation by AMT was higher in the presence of Res than in its absence, AA in combination with Res reduced the [^{14}C]DA formation below that observed with Res alone; the correlation between the stimulation of release and that of synthesis was positive with AMT but negative with AA.

The results discussed above were obtained after 10-min incubations (Table 1); 5- or 15-minute incubation periods led to essentially similar observations. In the absence of Res, 9.1 μM AMT or AA stimulated [^{14}C]DA synthesis at 5 min (AMT, 182.5 per cent and AA, 125.4 per cent of the control) and at 15 min (AMT, 221.3 per cent and AA, 185.7 per cent). Following the co-addition of Res, however, the AMT stimulation could be observed at 5 min (236.4 per cent of Res alone) and 15 min (242.4 per cent), but AA failed to stimulate and, in fact, reduced the [^{14}C]DA synthesis to 54.8 and 47.2 per cent at 5 and 15 min, respectively. In these incubations, however, both AMT and AA stimulated release in the absence (AMT indices, 1.94 and 2.89; AA indices, 1.21 and 2.41) as well as in the presence of Res (AMT, 1.35 and 1.60; AA, 1.23 and 1.34).

Our results in Table 1, therefore, suggest that Res sensitivity may differentiate the mechanism of action of AA from that of AMT. Res has been suggested [19] to release nerve ending vesicular catecholamines, and it has been shown that Res is a potent inhibitor of DA uptake by caudate nucleus synaptic vesicles [20]. Therefore, it is likely that the presence of Res in our incubation mixture impaired the retention as well as the uptake capacity of the vesicles for the catecholamines. A probable resultant higher cytoplasmic amine level, expressing itself by an inhibitory effect on [^{14}C]DA synthesis (Table 1), appears to be consistent with such Res action. The *in vivo* effect of Res (Table 2), which reduces striatal DA concentration to less than 10 per cent [16], is likely to affect strongly the uptake and retention capacities of the vesicles and deplete the storage catecholamines to a great extent. Our observed lack of any significant effect of AA on [^{14}C]DA synthesis (Table 2) would, therefore, suggest that synthesis stimulation (Table 1) by AA may be dependent upon an intact vesicular storage system for the catecholamines. An action of AA on the vesicular system is also consistent with the added AA inhibition observed in the presence of Res with untreated brain P_2 fraction (Table 1). It appears likely that this added inhibitory effect of AA, if due to an action on the vesicular system, differs distinctly from that of Res. With the synaptosomes from the Res-pretreated animals, AA was effective (Table 2) at 9.1 μM (and as low as 0.22 μM, data not shown) in stimulating the release of [^{14}C]DA but added Res failed to alter synthesis or release (Table 2). The stimulation of DA synthesis by AMT (Table 2) however, would suggest that this drug, unlike AA, may not require mediation of the vesicular storage system for its action.

Methylphenidate is another non-amphetamine central stimulant, and its behavioral effects, like those of AA, may be blocked by Res [21]. Considering that Res sensitivity also differentiates the stimulating effect of methylphenidate [18] on DA synthesis from that of AMT, it appears likely that the mechanism of action of AA suggested by our results is relevant to this other non-amphetamine as well.

In summary, our results indicate that AA may mimic AMT actions of stimulating synaptosomal synthesis of DA and its release. Our data also suggest, however, that Res action may help to distinguish AA from AMT with respect to their intrasynaptosomal effects. Although both AA and AMT may mobilize the intrasynaptosomal catecholamines and cause heightened DA synthesis, the pool of DA mediating the AA effects does not appear to be identical with that participating in AMT actions and may be vesicular in nature. Shore *et al.* [6, 22] has suggested that a mobilization of intrasynaptosomal storage DA to an impulse releasable pool is effected by AA, due to its inhibition of DA reuptake and a consequent decrease of the level of free cytoplasmic DA that inhibits mobilization. It is conceivable, however, that AA may have multiple effects, because it could, besides inhibiting reuptake, affect the intrasynaptosomal DA pools directly, with a consequent effect on the suggested mobilization process. An intrasynaptosomal action of AA of some nature is indeed suggested by our recent observation [23] of a preferential release of synaptosomal [^{3}H]DA over [^{14}C]DA, respectively, appearing from [^{3}H]dopa and [^{14}C]tyrosine. Furthermore, the added inhibitory effect of AA (Table 1) on the total DA formation with Res *in vitro* is most likely due mainly to an intrasynaptosomal action rather than to a reuptake inhibition, because the reuptake inhibition would lower the cytoplasmic DA level with a consequent opposite effect of increasing DA formation through a disinhibition of phenylalanine hydroxylation.

How an added inhibitory effect on DA synthesis by AA in combination with Res may be mediated by an intrasynaptosomal action of AA is not clear. One possible explanation is that any AA interaction with the storage vesicles, with their uptake and retaining capacities partially impaired by a short *in vitro* Res action, may lead to a further spilling of catecholamines. The results may be an enlarged cytoplasmic inhibitory amine pool acting on tyrosine hydroxylase. Further studies on AA effects are in progress.

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